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Supplementation of Dietary Nano Zn-Phytogenic on Performance, Antioxidant Activity, and Population of Intestinal Pathogenic Bacteria in Broiler Chickens

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ABSTRACT

Zinc is one of the essential minerals that are important for poultry. The disadvantage of Zn in the conventional form is its low bioavailability. One of the efforts to increase the bioavailability of Zn is to make it in a nano form. Nano Zn-Phytogenic (NZP), is a combination of Zn and phytogenic compounds of plants in nanoparticle size. The NZP was self-produced utilizing the green synthesis process of inorganic Zn and guava leave extract (*Psidium guajava*). The objective of this study was to evaluate the effects of supplementation NZP in diet on the performance, antioxidant status, and population of pathogenic intestinal bacteria (*Escherichia coli* and *Salmonella* sp) of broilers chicken. This study used 180 males and 180 females of Lohman broilers day old chick (DOC). The experiment was subjected to a completely randomized design with 6 (six) treatments and 5 (five) replications, and each experimental unit consisted of 12 DOCs (6 males and 6 females). The treatment given in this study were; R1= basal diet; R2= R1 + Zn Sulfate (90 mg Zn/kg) + 5.32 mg/kg guava leaf meal (added as a source of phytogenic compounds); R3= R1 + NZP (45 mg Zn/kg); R4= R1 + NZP (90 mg Zn/kg); R5= R1 + NZP (135 mg Zn/kg); R6= R1 + NZP (180 mg Zn/kg). The variables observed were performance, antioxidant activity in meat, and population of pathogenic intestinal bacteria (*E. coli* and *Salmonella* sp) of broiler chicken. The results showed that the addition of NZP up to a dose of 90 mg Zn/kg in the diet improved ($p<0.05$) body weight gain compared to the basal diet. The addition of NZP had no significant effect on the FCR. The addition of NZP increased ($p<0.05$) SOD activity in meat when compared with the dietary treatment without NZP. Groups of chicken fed NZP (R3, R4, R5, R6) had significantly ($p<0.05$) lower *E. coli* and *Salmonella* sp population. It could be concluded that the addition of NZP up to a dose of 90 mg Zn/kg in the diet of broiler chicken had positive benefits in improving performance, increasing antioxidant activity, and reducing pathogenic intestinal bacteria (*E. coli* and *Salmonella* sp).

***Keywords:** Nano Zn-Phytogenic; broiler chicken; performance; antioxidant; intestinal pathogenic bacteria*

INTRODUCTION

One of the essential minerals that are important for poultry is Zinc (Zn). It plays an important role in the metabolic process of protein and carbohydrates and in the process of growth and reproduction (Vinus & Sheoran, 2017; Chand *et al.*, 2014). Currently, the role of Zn is expanding as antioxidant, anti-heat stress, and antibacterial agents (Parashuramulu *et al.*, 2015; Zhao *et al.*, 2014). This new role of Zn would be beneficial in tropical countries such as Indonesia, which has a higher temperature than the ideal temperature for rearing broilers chicken. Especially at this time, the Indonesian government has implemented a ban on the use of antibiotic growth promoters (AGP) in animal feed.

High environmental temperature decreased the performance and immune response of broilers (Laudadio *et al.*, 2012). Heat stress supports the formation of reactive oxygen species (ROS) in excess amounts (Chand *et al.*, 2017). Accordingly, a balance between ROS production and the antioxidant system must be established (Saleh *et al.*, 2017). Dietary zinc (Zn) supplementation was reported to have a positive effect on the performance of growing poultry under heat stress conditions (Saleh *et al.*, 2018). This positive effect is because zinc is a fundamental element required for the structure and function of more than 300 enzymes related to metabolism (Salim *et al.*, 2011). Souza *et al.* (2019) report that Zn Nanoparticles have superior antibacterial activity against Gram-positive and Gram-negative pathogenic

bacteria. Xe *et al*. (2011) revealed that the possibilities of antibacterial mechanism of action of zinc oxide nanoparticles on membrane damage are caused by a direct or electrostatic interaction between ZnO and cell surfaces, cellular internalization of ZnO nanoparticles, and the production of active oxygen species such as H_2O_2 in cells due to metal oxides. The function of Zn nanoparticles as anti-bacteria is the same as the function of the antibiotic growth promoter (AGP) which suppress the growth of pathogenic bacteria.

It was reported, however, that the absorption of Zn in the digestive tract of broilers was very low (Swain *et al.,* 2012). Recently, one of the efforts to improve the bioavailability of Zn is developing nanoparticle of Zn (Fathi *et al.,* 2016a; Zhao *et al.,* 2014). Zhao *et al.* (2014) reported that Zn in the form of nanoparticles had a stronger chemical activity than in a conventional form and played a role in oxidation reactions with various organic compounds. Nano minerals also had the ability to cross the small intestine and subsequently enter the blood, brain, lungs, heart, kidneys, spleen, liver, intestines, and stomach (Bergin & Witzmann. 2013). Asheer *et al.* (2018) stated that Zn in nanoparticle size could improve growth, immunity, and reproduction and also acted as an antibacterial agent.

Nano Zn-Phytogenic (NZP) is a combination of Zn and phytogenic compounds of plants in nanoparticle size. The NZP was self-produced utilizing the green synthesis process of inorganic Zn and guava leaves extract *(Psidium guajava),* which acted as bioreductors and biostabilizers in the process of forming metal nanoparticles.

Guava leaves are reported to contain many active compounds, *i.e*., alkaloids, saponins, tannins, essential oils, flavonoids, and polyphenols (Pandey & Shweta, 2011; Sinurat *et al.,* 2018). Phytogenic compounds in guava leaf extract may have a function as feed additives, which will have positive benefits for the livestock body. Phytogenic compounds are potential to be used as an alternative growth promoter for broilers because they have the ability to improve gut health, performance, and immunity of broiler chicken without bacterial resistance (Murugesan *et al.,* 2015). Many studies showed that phytogenic compounds had been shown to improve production performance and immune responses in broiler chickens (Dhama *et al.,* 2015; Stanacev *et al.,* 2011; Zhang *et al.,* 2012; Zhou *et al.,* 2013).

Nano Zn-Phytogenic (NZP) is a new feed additive product that has been synthesized using an environmentally friendly nanotechnology process (green synthesis), with the main content of Zn and phytogenic (phenolic) compounds from guava leaf extract. NZP is expected to have the ability to promote growth, antibacterial, and antioxidant in broiler chickens. NZP is a source of Zn nanoparticles, and phytogenic compounds are expected to have positive benefits on antioxidant activity in broiler chickens. The active component of plants, namely polyphenols, was reported to have a strong antioxidant capacity (Chrpová *et al*., 2010). Furthermore, Perry *et al*. (2010) state that Zn is a cofactor of a major antioxidant enzyme, namely Cu/Zn Superoxide Dismutase (SOD). In other studies, it has been reported that the addition of Zn oxide nanoparticles is successful in increasing the antioxidant capacity of broilers, as evidenced by the increased Cu/Zn SOD activity and the decreased accumulation of malondialdehyde (MDA) in serum and liver (Zhao *et al*., 2014). Therefore, the main objective of this study was to evaluate the effects of NZP addition into the diet on the performance, antioxidant status, and population of pathogenic intestinal bacteria (*Escherichia coli* and *Salmonella* sp) of broiler chickens.

MATERIALS AND METHODS

This study was approved by the ethical clearance of the treatment and use of experimental animals from the Animal Welfare Commission of the Agricultural Research and Development Agency with the ethical clearance number: Balitbangtan/Balitnak/A/01/2019.

This study used 360 sexed day old chick (DOC) broilers of Lohman strain. The chicks were vaccinated with ND IB, ND Killed, IB Transume vaccine. The average body weight of male DOC was 48.44 g/bird, while the female DOC was 48.11 g/bird.

The cage used was the open house cage with a litter system with rice husks. The cage was provided with half-covered curtains to allow enough airflow. There were 30 cages with the size of each cage was 1.5 m x 1 m. Every cage was equipped with a feeder trough, drinking water, and brooder heater. Dietary treatments were formulated based on Rostagno *et al.* (2017) (Table 1).

Nano Zn-Phytogenic (NZP) has been characterized physically and chemically (Hidayat *et al.,* 2020) (in press). The phytogenic compounds in NZP were total phenol content from guava leaves. The process of making NZP was carried out by a green synthesis process in synthesizing metal nanoparticles. The main ingredients for making NZP were inorganic Zinc and guava leaf extract, forming complex bonds.

NZP treatment was added at doses equal to 0; 45; 90; 135; and 180 mg Zn/kg. Rations were given in the mash form for the ages of 1-7 days old and in the crumble for the ages of 8-33 days old. The experimental design used was a complete design with 6 (six) treatments and 5 (five) replications. Each experimental unit consisted of 12 DOCs (6 males and 6 females). Feed and drinking water were given *ad libitum*. The treatments design are presented in Table 2.

Management of Broiler Chicken

Broiler chickens were confined in cages that had fluctuating temperatures between morning, afternoon, and evening, ranging from 26ºC to 34°C. The ambient temperature was recorded at 6 AM, 12 PM; and 6 PM. The chickens were reared until the age of 33 days. The chickens weighing was carried out every week to determine body weight and to calculate body weight gain. The remaining feed provided was weighed once a week to calculate feed consumption.

Table 1. Composition and nutrient contents of the basal diet for pre-starter, starter, and finisher periods

Feedstuff	Prestarter (1-7 days old)	Starter (8-21 days old)	Finisher (22-33 days old)
Corn (%)	48.00	51.20	58.50
Soybean meal (%)	42.68	40	32.71
Palm oil (%)			4.60
Crude palm oil (%)	4.81	4.81	
$CaCO_3$ (%)	1.94	1.72	1.92
NaCl (%)	0.47	0.45	0.4
DL-Methionine (%)	0.31	0.28	0.25
Lysine (%)	0.28	0.2	0.28
Tricalcium phosphate (%)	1.35	1.17	1.17
Premix[a] (%)	0.17	0.17	0.17
Zinc sulfate (%)			0.001
Total	100	100	100
Nutrient contents[b]			
Crude protein (%)	24.57	23.55	20.67
Metabolizable energy (kcal/kg)	3018	3055	3155
Crude fat (%)	6.82	6.93	6.96
Crude fiber (%)	2.48	2.48	2.44
Lysine (%)	1.53	1.39	1.26
Methionine (%)	0.61	0.57	0.51
Methionine + Cysteine (%)	0.92	0.88	0.88
Calcium (%)	1.04	0.93	0.99
Available phosphorus (%)	0.47	0.43	0.42
Na (%)	0.23	0.22	0.20
Cl (%)	0.33	0.32	0.29
Zn (ppm)	41.29	40.06	40.02

Note: [a]Provides per kilogram of diet : vitamin A 15,000 IU; cholecalciferol, 3,900 IU; vitamin E 30 IU; vitamin K 3.0 mg; thiamine 2.4 mg; riboflavin, 9.0 mg; vitamin B6, 4.5 mg; vitamin B12, 0.021 mg; calcium pantothenate, 30 mg; niacin, 45 mg; folic acid 1.2 mg; biotin, 0.18 mg; choline (as choline chloride), 700 mg; Cu, 8 mg; Mn, 100 mg; Fe, 80 mg; I, 0.35 mg; Se, 0.15 mg.
[b]Obtained using the calculation method based on the nutrient content reported by the laboratory analysis of the Indonesian Research Institute for Animal Production, except for metabolic energy based on Rostagno *et al.* (2017).

Table 2. Nano Zn-Phytogenic (NZP) experimental diets

Treatments	Description	Total Zn on diet (mg/kg)
R1	Basal diet (Zn content according to NRC (1994); 40 mg/kg).	40
R2	R1 + (90 mg/kg Zinc Sulfate (conventional Zn) + 5.32 mg/kg guava leaf flour (adding as a source of phytogenic compounds)). Phenol content 0.63 mg/kg.	130
R3	R1 + NZP (45 mg Zn/kg), Phenol content 0.32 mg/kg	85
R4	R1 + NZP (90 mg Zn/kg), Phenol content 0.63 mg/kg	130
R5	R1 + NZP (135 mg Zn/kg), Phenol content 0.94 mg/kg	180
R6	R1 + NZP (180 mg Zn/kg), Phenol content 1.26 mg/kg	220

Note: The NZP used in the feeding trial contained 6.12% Zn and 430 mg/kg of total phenol. Guava leaves used contained 11.85% of total phenol.

Observation of Broiler Chicken Performance

Feed consumption (g/bird) was measured based on the difference between the ration given and the remaining rations left in the feeder trough, divided by the number of chickens in one cage. Body weight (g/bird) was measured by weighing the chickens in each cage each week, divided by the number of the chicken. Body-weight gain (g/bird) was calculated by subtracting the final total body weight by the total initial body weight and divided by the number of chickens. The feed conversion ratio was measured by dividing the average feed consumed by the average body-weight gain.

Analysis of meat Superoxide Dismutase (SOD)

Measurement of Superoxide Dismutase (SOD) enzyme activity in meat followed the procedure described by Martin Jr *et al.* (1987). The sample was prepared by weighing 1 g of fresh thigh meat, and put in a tube, added 2 mL of Phosphate Buffered Saline (PBS), homogenized and then centrifuged at 10000 rpm for 20 minutes. The supernatant was transferred into another tube as a sample for the next analysis. SOD levels were determined biochemically using the RanSOD® kit. Reagents in this kit consisted of a mixed substrate containing xanthine, phosphate buffer to

dilute (both standard and sample), xanthine oxidase, and standard solution (CAL). The reagents were then used to make a standard curve. At first, 25 μL of the sample was put into the cuvette, and 850 μL of the mixed substrate was added and mixed well. To inhibit SOD, 5 mL of sodium cyanide was added to the mixture until it was well mixed. After that, 125 μL of xanthine oxidase was added. The light absorption rate was read at a wavelength of 505 nm with a GENESYS 10S UV-Vis Spectrophotometer-Printer|Thermo Scientific ex. USA. Superoxide dismutase levels were determined using equations obtained from the standard curves.

Measurement of Meat Malondialdehyde (MDA)

Meat sample preparation was carried out by following the methods of Jung *et al.* (2016) with some modifications. Homogenate of chicken meat for MDA analysis was made by using 1 g of ground meat in cold conditions then added with 2 mL of PBS solution (1.15 g KCl in 100 mL PBS) with a pH of 7.4 and homogenized. The resulting homogenate was centrifuged at 10000 rpm for 20 minutes. The supernatant was taken and immediately stored at -20^{o} C. The measurement of MDA level was then carried out by the method of thiobarbituric acid-reactive substance (TBARS). The procedure was initiated by preparing a standard stock solution of MDA in aquabidest with seven different concentrations (standard blank, 0.25-1.6 mol). The 2 μL sample was put into a centrifuge tube and added 1800 μL of aquabidest, and 1000 μL of 20% TCA, and 2000 μL of TBA 0.67%. Then the mixture was heated at 95°C for 10 minutes. The solution was allowed to reach room temperature, and then it was centrifuged at 3000 rpm for 10 minutes. The procedure was also applied to the blank. The supernatant was carefully taken and then measured for its absorption with a GENESYS 10S UV-Vis Spectrophotometer-Printer|Thermo Scientific ex. USA at a wavelength of 530 nm. MDA standard curves were made with a concentration of 0 nmol; 0.0125 nmol; 0.025 nmol; 0.05 nmol; 0.1 nmol; 0.4 nmol; 1.6 nmol; and 32 nmol in 2000 μL. The sample MDA level was calculated by using the standard curve.

Measurement of *Escherichia coli* and *Salmonella* sp Population in the Intestine

Concentrations of *E. coli* and *Salmonella* in the intestinal digesta were measured by taking the sample of digesta from the ileum portion of the selected bird from each treatment replication. The digesta was removed from the ileum, then put in a sterile plastic, and placed in a cool box for the next laboratory analysis. Calculations of *E. coli* and *Salmonella* sp concentrations were carried out using a dilution method according to Balouiri *et al.* (2016); 1 g of sample was diluted with 9 mL of KH_2PO_4 solution. Dilution was carried out from 10^{-1}-10^{-4}, then from each dilution, 1 mL of solution was pipetted and inserted into a petri dish that has been coded in duplicate. Furthermore, Eosin Methylene Blue Agar (EMBA) was poured, homogenized, and then incubated for 24-48 hours with a temperature of 37^{o}C.

Data Analysis

Data were analyzed statistically using a variety of tests (ANOVA). Firstly, the data were tested for normality. The normal data were then analyzed with ANOVA, if there were significant ($p<0.05$) differences the analysis was continued for Duncan's test. The polynomial test was performed on body-weight gain data in order to determine the optimum dose of NZP. Some variables (meat antioxidant activity, populations of *E. coli* and *Salmonella* sp on intestinal (*ileal*) digesta) were tested by an orthogonal contrast to see the effect of with and without NZP addition treatment excluded treatment 2 (R2).

RESULTS

Temperature and Humidity Conditions during Broiler Maintenance

Observing the effect of NZP addition in broiler diet involves the recording of temperature and humidity of the air around the experimental cage, and the figures are presented in Table 3. The chickens were exposed to heat from the environment for ± 12 hours at temperatures ranging from 25.2-31.5°C and humidity 49.52%-72.6%.

Addition of Nano Zn-Phytogenic (NZP) on the Performance of Broiler Chickens

The average consumptions of Zn and phenol (mg/bird) for the treatment groups are presented in Table 4. Meanwhile, the Scatterplot and regression lines of the effect of increasing dose (mg/kg) of NZP on body weight gain (g/bird) of the chickens are presented in Figure 1. The addition of NZP had a significant effect ($p<0.05$) on feed consumption. The use of NZP up to a dose of 135 mg/kg did not influence feed consumption compared to R1 (without the addition of NZP) and R2 (addition of conventional Zn). Feed consumption decreased when adding NZP at a dose of 180 mg Zn/kg (R6). Body weight gain (BWG) and body weight (BW) of the chickens were affected ($p<0.05$) by the addition of NZP. BWG and BW for the treatment group, given the addition of NZP at doses of 45 and 90 mg Zn/kg did not differ significantly from the treatment group that was fed conventional Zn at a dose of 90 mg Zn/kg (R2). The addition of NZP at a dose of 180 mg Zn/kg decreased BWG and BW. The addition of NZP had no significant effect on the FCR. In this study, chickens in R3 treatment diet (45 mg Zn/kg dose from NZP) showed relatively similar FCR to chickens fed R2 treatment diet (90 mg Zn/kg of conventional Zn).

Addition of Nano Zn-Phytogenic (NZP) on Meat Antioxidant Activity

The effect of adding NZP on the meat antioxidant activity (Superoxide Dismutase/SOD) and lipid oxidation product (Malondialdehyde/MDA) are presented in Table 5. The addition of NZP increased ($p<0.05$) SOD activity when compared with the control group (R1) without the addition of NZP. This result was also shown

Table 3. Temperature and relative humidity in the animal house during the course of the experiment of dietary Nano Zn-Phytogenic (NZP) in broiler chickens

Week	Morning (6 AM)		Noon (12 PM)		Afternoon (6 PM)		Recommended[1)]	
	Temperature (°C)	Relative humidity (%)	Temperature (°C)	Relative humidity (%)	Temperature (°C)	Relative humidity (%)	Temperature (°C)	Relative humidity (%)
1	26.94	61.00	34.97	45.57	33.51	41.57	27-30	60-70
2	26.94	72.29	30.96	59.29	31.10	59.00	25-26	60-70
3	22.07	73.43	29.86	49.43	30.37	48.29	22-24	60-70
4	23.97	77.86	31.29	51.29	31.13	51.29	20-21	60-70
5	20.03	64.66	26.24	41.59	26.75	40.37	20-21	60-70
Average	25.00	72.06	31.70	50.94	31.50	49.52		
SEM	0.48	1.48	0.41	1.23	0.35	1.92		

Note: [1)]Lohman (2009)

Table 4. Performance of broiler chicken fed experimental Nano Zn-Phytogenic (NZP) diet up to the age of 33 days

Treatment	Variables					
	Feed consumption (g/bird)	Body weight gain (g/bird)	Body weight (g/bird)	FCR	Zn consumption (mg/bird)	Phenol consumption (mg/bird)
R1	2,164.20^{a}	1,257b^{c}	1,307^{ab}	1.67	86.57	0
R2	2,291.40^{a}	1,384^{a}	1,432^{a}	1.58	297.89	1.45
R3	2,076.80^{ab}	1,310^{ab}	1,357^{a}	1.58	176.53	0.66
R4	2,235.70^{a}	1,352^{ab}	1,401^{a}	1.64	290.65	1.41
R5	2,053.10^{ab}	1,259^{bc}	1,307^{ab}	1.56	359.29	1.95
R6	1,851.30^{b}	1,176^{c}	1,224^{b}	1.57	407.29	2.34
SEM	44.30	19.43	20.17	0.017		
p-value	0.0452	0.0116	0.0118	0.412		

Note: R1= basal diet; R2= R1 + Zn Sulfate (90 mg Zn/kg) + 5.32 mg/kg guava leaf flour; R3= R1 + NZF (45 mg Zn/kg); R4= R1 + NZF (90 mg Zn/kg); R5= R1 + NZF (135 mg Zn/kg); R6= R1 + NZF (180 mg Zn/kg). Means in the same column with different supperscripts differ significantly (p<0.05).

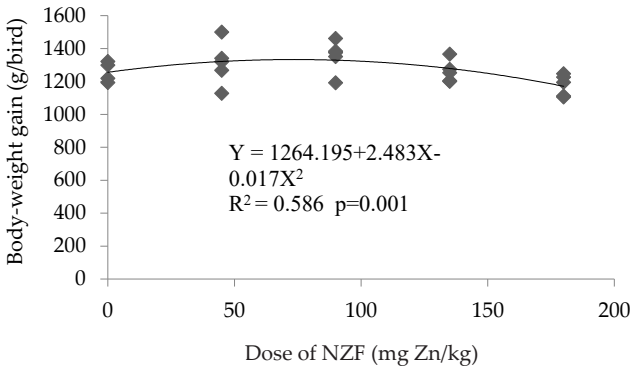

Figure 1. Scatter plot and regression line of body weight gain of broiler chickens fed experimental Nano Zn-Phytogenic (NZP) diet

by the significant (p<0.05) effect of the orthogonal contrast test (Table 5) between groups without the addition of the NZP treatment (R1) and groups with the addition of NZP (R3, R4, R5, R6). The addition of NZP in the diet significantly (p<0.1) reduced lipid oxidation (Malondialdehyde/MDA) in meat as were shown by the results of the orthogonal contrast analysis between the control group without the addition of NZF (R1) and groups with the addition of NZP (R3, R4, R5, R6).

Population of *Escherichia coli* and *Salmonella* sp in the Intestine

The effects of dietary NZP on the populations of *E. coli* and *Salmonella* sp in the intestinal digesta (*ileum*) are presented in Table 6. The addition of NZP significantly (p<0.05) decreased the number of *E. coli* compared to the control group without the addition of NZP (R1). The addition of NZP at a dose of 90 mg Zn/kg resulted in the lowest number of *E. coli* bacteria compared to other treatments. The addition of NZP was also able to reduce the number of *Salmonella sp* bacteria in the intestine compared to the control group without the addition of NZP (R1) and the treatment group that was given the addition of conventional Zn at a dose of 90 mg Zn/kg (R2). The orthogonal contrast test showed that chickens in groups R3, R4, R5, and R6 significantly (p<0.01) decreased *Salmonella sp* population compared to the control group without the addition of NZP (R1). The addition of NZP at a dose of 90 mg Zn/kg resulted in the lowest amount of *Salmonella sp* bacteria compared to other treatments.

DISCUSSION

Broiler Chickens Performance

The addition of NZP at a dose of 180 mg/kg reduced feed palatability. This effect was thought to be re-

Table 5. Antioxidant activity (Superoxide Dismutase/SOD) and lipid oxidation products (Malondialdehyde/MDA) in thigh meat of broilers chickens fed experimental Nano Zn-Phytogenic (NZP) diet up to the age of 33 days

Treatments	Variables	
	SOD (U/mL)	MDA (nmol/mL)
R1	14.5^{c}	0.46^{a}
R2	21.9^{ab}	0.13^{b}
R3	22.8^{ab}	0.15^{b}
R4	19.6^{bc}	0.16^{b}
R5	21.1^{ab}	0.13^{b}
R6	26.5^{a}	0.19^{ab}
SEM	1.05	0.039
p-value	0.031	0.104
Contrast orthogonal p-value:		
R1 vs R3, R4, R5, R6	0.010	0.059

Note: R1= basal diet; R2= R1 + Zn Sulfate (90 mg Zn/kg) + 5.32 mg/kg guava leaf flour; R3= R1 + NZF (45 mg Zn/kg); R4= R1 + NZF (90 mg Zn/kg); R5= R1 + NZF (135 mg Zn/kg); R6= R1 + NZF (180 mg Zn/kg). Means in the same column with different supperscripts differ significantly ($p<0.05$).

Table 6. Populations of *Eschericia coli* and *Salmonella* sp on intestinal (ileal) digesta of broiler chickens fed experimental Nano Zn-Phytogenic (NZP) diet up to the age of 33 days

Treatments	Population	
	Escherichia coli (log CFU/g)	*Salmonella* sp (log CFU/g)
R1	4.23^{A}	2.39^{A}
R2	2.91^{AB}	1.89^{B}
R3	3.56^{A}	1.13^{C}
R4	1.00^{C}	1.00^{C}
R5	1.47^{BC}	1.09^{C}
R6	3.28^{A}	1.19^{C}
SEM	0.30	0.12
p-value	0.0023	<0.0001
Contrast orthogonal p-value:		
R1 vs R3, R4, R5, R6	0.004	<0.0001

Note: R1= basal diet; R2= R1 + Zn Sulfate (90 mg Zn/kg) + 5.32 mg/kg guava leaf flour; R3= R1 + NZF (45 mg Zn/kg); R4= R1 + NZF (90 mg Zn/kg); R5= R1 + NZF (135 mg Zn/kg); R6= R1 + NZF (180 mg Zn/kg). Means in the same column with different supperscripts differ significantly ($p<0.01$).

lated to the cumulative consumption of Zn and phenols that enter the body of broilers chickens. Table 4 showed the consumption of Zn and phenol (mg/bird) of the experimental chickens for each treatment group during the observation period. The chickens in the R6 treatment consumed a total of 407.29 mg of Zn/bird and consumed a phenol of 2.34 mg/bird. Ahmadi *et al.* (2013) reported that feed consumption decreased significantly with the addition of nano ZnO in the diet of broiler chicken at doses of 60 and 90 mg/kg. Mahmoud *et al.* (2013) reported that the addition of 1% guava leaves in the diet of broiler chicken reduced feed consumption. Meanwhile, Starcevic *et al*. (2015) reported that phenol compounds in the form of tannins exhibited anti-nutritional and toxic effects that would depend on their intake and bioavailability. The results of the study indicated that the cumulative consumption of Zn and phenol compounds in high doses was one of the factors in decreasing feed consumption in broiler chickens. In the present study, feed consumption ranged from 1851-2291 g/bird, which was lower than the normal feed consumption of broiler chickens as suggested by Lohman (2009) (more than 2611 g/bird).

The ambient temperature around the cage reached 33°C during the day, and this high environmental temperature was the main factor that probably caused the low feed consumption. High air temperatures were reported to reduce feed consumption of broiler chickens (Syafwani *et al.,* 2011). The reduced feed consumption was due to the adjustment of body's temperature of the chicken to the environment temperature by suppressing endogenous heat production through the reduction of feed consumption. Syafwani *et al.* (2011) also stated that in order to increase survival rates under high ambient temperatures, broilers chicken reduced feed consumption in order to reduce metabolic heat production, which eventually would reduce body weight.

Achievement of body weight (BW) at 33 days (Table 4) of NZP treatment at a dose of 45 mg Zn/kg (R3) was similar to the body weight of the group of broiler chickens fed ration supplemented with conventional Zn at a dose of 90 mg Zn/kg (R2). This result indicated an equal level of Zn efficiency when given in the form of NZP. The relationship between NZP doses and the body weight gain (BWG) was expressed in a regression formula of $Y= 1264.195 + 2.483X-0.017X2$ (Figure 1), showing that the optimum dose of dietary NZP producing the highest BWG was 73 mg Zn/kg. The optimum dose of NZP addition was actually lower than the results obtained from a meta-analysis study, which was 90 mg of non-nano particles Zn/kg to reach the highest BWG (Hidayat *et al.,* 2020).

These results indicated that the size of Zn particle changing to nanoparticle as in NZP products reduced the dose of Zn use in the feed, which might impact on increasing Zn bioavailability. Some reports indicated that the addition of Zn nanoparticles in the ration of broiler chickens had a positive effect on the increasing body weight of broiler chickens. Zhao *et al*. (2014) reported that the addition of 20 and 60 mg nano-Zn/kg ration in broiler chickens increased body weight. Fathi *et al.* (2016b) also reported that the addition of nano Zn in the form of ZnO at a dose of 20 mg/kg significantly increased body weights of broiler chickens. This result showed that reducing the size of Zn particle had a positive effect on the growth of broiler chickens. Mohammadi *et al*. (2015) stated that the application of nanotechnology helped to provide Zn more efficiently. Particles in nanosize could by-pass conventional physiological pathways from the distribution and transportation of nutrients across tissues and cell membranes and protected Zn from the destruction process before reaching the target (Asheer *et al*., 2018).

In this study, the addition of NZP at doses of 45 and 90 mg Zn/kg ratio had a positive effect on body weight gain. The positive effects of NZP addition, not only as an effect of Zn but also the effects of phytogenic compounds bound in NZP. Previously, several researchers (Stanacev *et al*., 2011; Dhama *et al*., 2015) reported that the use of phytogenic feed additives in the diet improved the performance of poultry. Mahmoud *et al.* (2013) also reported that phytogenic compounds obtained from guava leaves increased body weight gain of broiler chickens. Phytogenic feed additive improved the growth of broiler chickens due to the increase in the utilization of nutrients, stimulation of digestive enzymes (i.e., lipase, amylase, or protease), and improved microbiota ecosystem by controlling pathogenic bacteria in the digestive tract (Hashemi & Davoodi, 2011).

However, on the contrary to the positive effects, the addition of NZP up to 180 mg Zn/kg ration decreased BWG. Zhao *et al.* (2014) reported that Zn nanoparticles had a toxic effect if used in a high dose. Siddiqi *et al*. (2018) stated that Zn in the form of nanoparticles was much more active and could be quickly converted into ions in the stomach. This condition will cause the production of large amounts of metal ions and then be brought into the liver and kidneys for metabolism and excretion, which eventually caused damage of the liver and kidney tissues (Zhao *et al*., 2014). In addition, Zn in the form of nanoparticles was able to increase the biocompatibility of Zn against cells (Wahab *et al*., 2016). Some researchers (Lu *et al*., 2013; Boverhof *et al*., 2015) expressed concern about the possible negative effects of the use of nanoparticle material. Recently, acute toxicity of the use of nano-ZnO had been investigated either *in vitro* or *in vivo* (Sharma *et al*., 2012; Esmaeillou *et al*., 2013; Setyawati *et al*., 2015). The toxicity of nano-ZnO increased with a decrease in size as well as an increase in concentration (Yan *et al*., 2012; Lopes *et al*., 2014). Body weight and organ weight are general and sensitive indicators for identifying the harmful effects of drugs on animals (Nirogi *et al*., 2014). Jeevanandam *et al.* (2018) explained that nano minerals, despite being important supplying micro minerals, were actually toxic at a higher rate than the required dosage.

FCR of chickens fed diet supplemented with NZP were lower than those fed ration without NZP (R1). This result indicates that the addition of NZP has a positive benefit in increasing the efficiency of feed utilization. Some references reported that the addition of Zn nanoparticles provided a positive response to FCR. Ahmadi *et al*. (2013) stated that FCR was improved significantly through the addition of ZnO nanoparticles at doses of 60 and 90 mg/kg. Similarly, Zhao *et al.* (2014) reported that the addition of ZnO nanoparticles to the basal diet produced better FCR at doses of 20 and 60 mg/kg. Meanwhile, Fathi *et al*. (2016a) found better feed efficiency in the addition of nano ZnO at a dose of 20 mg/kg in the basal diet.

Antioxidant Activity

The antioxidant activity of SOD caused by dietary NZP at a dose of 45 mg Zn/kg (R3) was not statistically different from that caused by a diet with conventional Zn at a dose of 90 mg Zn/kg. Zn is actually a cofactor for SOD and has an important function in antioxidant systems, such as an inhibitor of the oxidation process by protecting proteins and enzymes and as an inhibitor of free radical formation (Yuan *et al*., 2011). SOD is widely distributed and protects various organs and tissues from peroxidation (Fukai & Fukai, 2011). The important role of Zn in immune response activities is related to the influence of Zn on the antioxidant defense mechanisms in the body (Lee, 2018). Marreiro *et al.* (2017) stated that Zn increased antioxidant activity by reducing the production of free radicals because Zn competes with the other minerals, such as copper and iron, in binding to the cell membranes.

The addition of NZP to the feed had a tendency to be able to inhibit lipid peroxidation. MDA is a product of the lipid oxidation process. A high MDA value indicated a high oxidized lipid value. Lipid oxidation is related to the level of antioxidant activity. Zn plays an important role in antioxidant activity, having an impact on reducing the lipid oxidation process (Hidayat *et al*., 2020). Lee (2018) explained that as a cofactor of many anti-oxidative enzymes, Zn plays a key role in reducing the production of free radicals. Previously, several researchers (Marreiro *et al*., 2017; Lee, 2018) reported that Zn was able to reduce MDA, which showed that Zn functioned as an antioxidant agent, reducing lipid peroxidation in the cell membranes. The positive effects of the addition of NZP are the increased antioxidant activity of SOD enzymes and having the ability to inhibit the occurrence of lipid peroxidation (MDA) in broiler chickens. Besides showing a positive benefit due to Zn content, NZP also has a positive effect due to its phytogenic elements contents. Lee *et al.* (2016) stated that plant phytogenic elements (phenols) had an antioxidant capacity. Meanwhile, Chrpová *et al.* (2010) suggested that the active component of plants, namely polyphenols, also had a strong antioxidant capacity. Hydrolyzed tannins, as part of polyphenols, are also reported to play a role as a powerful source of antioxidants (Mojzer *et al*., 2016). Moreover, guava leaves that were used in the NZP synthesis process had the potential as a source of antioxidants (Lee *et al*., 2012; Rivai *et al*., 2010). Bintarti (2014) also reported that guava leaf extract had antioxidant activity in a strong category.

Populations of *Escherichia coli* and *Salmonella* sp Bacteria in the Intestine

Nanoparticles were reported to have strong abilities in bacteriostatic (inhibiting the growth) and bactericides (killing the bacteria) (Arabi *et al*., 2012). *E. coli* and *Salmonella* sp are pathogenic bacteria that can be found in the intestines of animals (Iovine *et al*., 2015). Kemmett *et al.* (2014) stated that *E. coli* is the main factor in the mortality of the newly hatched chicks, which contributes to economic losses in the poultry industry. The addition of NZP at a dose of 90 mg Zn/kg resulted in the lowest number of *E. coli* bacteria compared to the other treatment groups. The results of the orthogonal contrast test between the treatment group without NZP (R1)

and with NZF (R3, R4, R5, R6), showed that there were significant differences ($p<0.05$) in the *E. coli* population in the intestine. The significant ($p<0.01$) reduction in the number of *Salmonella* sp bacteria in the intestine was shown in chickens fed a diet containing NZP compared to the group fed control diet without NZP (R1) or the chickens that were fed a diet contained a conventional Zn at a dose of 90 mg Zn/kg ration. This condition was also confirmed by the orthogonal contrast test results, comparing the variable in the group of the chicken fed diet without NZP (R1) with the group of chickens fed the diet with NZP (R3, R4, R5, R6). The addition of NZP at a dose of 90 mg Zn/kg showed the lowest number of *Salmonella* sp bacteria compared to the other treatment groups. This result indicated that NZP had the potential to be developed as an antibacterial agent in broiler chicken's diet.

Seil & Webster (2012) explained that nanoparticles had a greater surface area available to interact with the surface of bacteria to enhance the bactericidal effect as compared to large particles. Slavin *et al*. (2017) reported that nanomaterials released ions reacted with thiol groups (-SH) from proteins present on the cell surface. The protein protrudes through the cell wall, facilitate and allow the transportation of nutrients. Zn nanoparticles deactivate proteins, reduce membrane permeability, and ultimately cause cell death (Rajendran *et al.,* 2010). Furthermore, Lahir (2020) added that minerals in the form of nano also was slowing the attachment of bacteria and inhibiting the formation of biofilms. The specific mode of action of nanoparticles against bacteria made it an ideal candidate as an antimicrobial agent without the risk of developing bacterial resistance (Arabi *et al*., 2012). The antibacterial activity shown by NZP was also caused by the activity of phytogenic compounds derived from guava leaf extracts incorporated in the NZP. The results of many studies indicated that phytogenic elements contained in guava leaves (flavonoids, gallocatechin) had the ability as an antibacterial, including against *E. coli* and *Salmonella* sp (Biswas *et al*., 2013). Phytogenic character of guava leaves (*Psidium guajava* L) was reported by Biswas *et al*. (2013) as a part of a plant containing antimicrobial compounds, *i.e*., tannins, essential oils (eugenol), fatty oils, resins, triterpenoids, flavonoids, and malic acid. Antimicrobial compounds in guava leaves had the ability to suppress Gram-positive and Gram-negative bacteria (Biswas *et al*., 2013).

CONCLUSION

Supplementation of Nano Zn-Phytogenic (NZP) up to a dose of 90 mg Zn/kg in the broiler chicken diet had positive benefits in improving performance, increasing antioxidant activity, and functioning as an antibacterial against pathogenic bacteria (*Escherichia coli* and *Salmonella* sp) in the intestine.

CONFLICT OF INTEREST

Anuraga Jayanegara and Elizabeth Wina serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The Authors also certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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